irradiation [5]. One type of stabilizing action by cepharanthine on the biological membrane and the artificial lipid bilayer is by this mechanism. (2) Cepharanthine prevented Na^+, K^+-ATPase inhibition caused by ascorbate alone in the absence of Fe^{3+}. Since ascorbate radicals are responsible for the Na^+, K^+-ATPase inhibition under these conditions [6], it is tempting to conclude that the alkaloid may protect the membrane enzyme from ascorbate radicals although the exact mechanism is unclear. The alkaloid is reported to have no ability to scavenge free radicals [5], and hence the mechanism is unlikely to involve the elimination of ascorbate radicals by cepharanthine. Whether the drug inhibits radical formation itself is unknown; no data are available in the literature. The cepharanthine protection may also not be through the mechanisms that are relevant to the inhibition by alcohol, ouabain, and SH reagents, inasmuch as the inhibition by these agents was unaffected by the presence of the alkaloid (Table 2). Thus, cepharanthine may exert its action of sheltering biologically active membranes by protection not only of the lipid component but also of the enzyme component.

In summary, cepharanthine, a biscoclaurine-type alkaloid obtained from *S. cephalantha*, was investigated for its protective action of Na^+, K^+-activated ATPase of the plasma membranes from rat cerebral synaptosomes. The results indicate that there might be two types of mechanisms of cepharanthine action: the alkaloid prevented both membrane lipid peroxidation, which in turn inhibited the ATPase integrated in the lipid bilayer, and the deleterious effects of ascorbate, whose effective form was its radical, although the exact mechanism remains to be clarified.

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Binding of phospholipase A_2 to isolated heart muscle

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Phospholipases A_2 (EC 3.1.1.4) isolated from snake venoms vary in lethal potency and enzymatic activity [1, 2]. They are used extensively as enzymatic probes with which to study phospholipid function and organization in membranes [1, 3]. Many basic snake venom phospholipases A_2 have potent pharmacological effects while other venom phospholipases A_2, usually acidic or neutral, are much weaker in their pharmacological properties [1, 2, 4–6]. However, the acidic or neutral phospholipases have enzymatic activities, as measured in various *in vitro* systems, that are often much greater than those of the basic enzymes [1, 2, 4–6]. Even though there is a lack of correlation between pharmacological and enzymatic activities, it has been suggested that the pharmacological properties of phospholipases A_2 are due to phospholipid hydrolysis [1]. Our previous studies with native and chemically modified derivatives of the basic *Naja nigricollis* phospholipase A_2 and the acidic *Naja naja atra* phospholipase A_2 have shown that the extent of phospholipid hydrolysis, as measured directly in the tissue being studied, does not correlate with their pharmacological properties [4–12]. These results led us to conclude that some pharmacological effects of snake venom phospholipases A_2 are due to a non-enzymatic action.

The phospholipase A_2 from *N. nigricollis* venom has potent cardiotoxic effects which do not correlate with the very low levels of tissue phospholipid hydrolysis or production of phospholipid hydrolytic products [4–6,*]. The *N. n. atra* phospholipase A_2, in contrast, shows little cardiotoxicity even though it causes the same low level of phospholipid hydrolysis [4–6,*] as the *N. nigricollis* enzyme. Since cardiotoxicity appears to be due to a non-enzymatic action of this phospholipase A_2, it was of interest to investigate whether differences in potency correlated with the extent of binding to heart tissue.

* Unpublished results.

Materials and methods

Lyophilized *N. nigricollis* snake venom was obtained from the Miami Serpentarium Laboratories (Miami, FL), and lyophilized *N. n. atra* snake venom was collected in Kaohsiung, Taiwan, by one of the authors (C. C. Y.). The methods for purification and homogeneity determination of the most basic (pI 10.6) phospholipase A_2 from *N. nigricollis* snake venom and the major acidic (pI 5.2) phospholipase A_2 from *N. n. atra* snake venom are identical to those previously described [13, 14].

Radiolabeled *N. nigricollis* and *N. n. atra* enzymes were prepared by the iodine monochloride (Sigma) method of MacFarlane [15] as modified by Vogel *et al.* [16]. The specific activity of the labeled *N. n. atra* enzyme was 44.2 Ci/mmole protein. To obtain adequate amounts of material and to perform all experiments within 3 weeks following iodination, it was necessary to prepare two iodinated batches of *N. nigricollis* phospholipase A_2 (sp. act. of 14.1 and 0.87 Ci/mmole protein).

Following iodination (up to 4 weeks), these phospholipases retained 50–100% of their enzymatic activity (measured on lecithin-Triton mixed micelles) and 50–100% of their original lethal potency (intraventricular injection into rats).

Hearts from male Sprague–Dawley rats (150–300 g), obtained from the Charles River Breeding Laboratories (Wilmington, MA), were rapidly removed under ether anesthesia. The hearts were rinsed twice in Tyrode's solu-

tion [17] or, where binding was to be measured in the absence of phospholipase A_2 enzymatic activity, in 5 mM EDTA Tyrode's solution without Ca^{2+}. The right ventricular muscle was used in these binding studies since this preparation had been used previously in our electrophysiological studies on the cardiotoxic action of *N. nigricollis* phospholipase A_2 [4–6]. The right ventricular wall was separated and placed in a 20-ml beaker containing 4 ml of Tyrode's solution bubbled with O_2 and CO_2 (95:5) at room temperature. After a 30-min equilibration period, the bathing solution was replaced with one of the following: fresh Tyrode's solution, the non-radioactive phospholipase A_2 in the appropriate Tyrode's solution (normal Tyrode's or 5 mM EDTA Tyrode's solution) or detergent in 5 mM EDTA Tyrode's solution. After an additional 5-min equilibration period, either [^{125}I]-*N. nigricollis* phospholipase A_2 or [^{125}I] *N. n. atra* phospholipase A_2 (5.4×10^{-9} M) was added. At the completion of the incubation period (60 min), the ventricular wall was rinsed in 30 ml of Tyrode's solution, blotted, weighed and counted for 10 min using a Packard Gamma Counter model 5110, with the results being recorded as cpm per min.

Parallel experiments to the binding experiments, except for the addition of only non-radioactive phospholipase A_2, were used to measure phospholipid hydrolysis. Following exposure to phospholipase A_2, hearts were homogenized, using a Polytron homogenizer, in 1.5 to 2.0 ml of a 10 mM EDTA solution to stop enzymatic activity [18]. The homogenates were extracted with 1:3 chloroform–methanol [19] followed by 2:1 chloroform–methanol [20], and the phospholipids were separated by two-dimensional thin-layer chromatography [21]. Individual phospholipids were detected with iodine vapor and ninhydrin spray, and the phosphorus content of each separated spot was measured [22]. Phospholipid hydrolysis was estimated from the phosphate values in the lysophospholipid spots and the decrease in phosphate values of the phospholipid spots.

The data are presented as means ± standard error. The statistical significance of differences was determined by Student's *t*-test.

Results and discussion

The binding of 5.4×10^{-9} M [^{125}I] *N. n. atra* phospholipase A_2 in normal Tyrode's solution was decreased 57% by exposure to a 100-fold excess of non-radioactive *N. n. atra* phospholipase A_2 (Table 1). The values for binding of *N. nigricollis* and *N. n. atra* phospholipase are similar (Table 1), suggesting that differences in binding were not responsible for the marked differences in cardiotoxic potencies of these two enzymes. Also, preliminary unpublished observations, using conditions identical to those in this study, suggested that *N. nigricollis* and *N. n. atra* phospholipase A_2 bind to overlapping sites since a 100-fold excess of one enzyme displaced 28–33% of the other enzyme (N = 2). In marked contrast to these results, however, exposure to a 100-fold excess of non-radioactive *N. nigricollis* phospholipase A_2 more than doubled the binding of the radioactive *N. nigricollis* enzyme. This effect was observed in experiments carried out in normal Tyrode's solution or more significantly in a 5 mM EDTA Tyrode's solution (free of Ca^{2+}), in order to block enzymatic activity (Table 1). This unexpected ability of 5.4×10^{-7} M *N. nigricollis* phospholipase A_2 to increase the binding of a 5.4×10^{-9} M concentration of the radioactive enzyme did not appear, therefore, to be dependent upon the enzymatic activity of the enzyme. Our phospholipid measurements on the tissue are in agreement with this conclusion. Following exposure of hearts to Tyrode's solution containing 5.4×10^{-7} M *N. nigricollis* phospholipase A_2, it was observed that 9.8 ± 1.8 (mean ± S.E.) and 7.8 ± 1.7% (N = 3) of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), respectively, were present as lysophospholipids while the corresponding levels of hydrolytic products in the 5 mM EDTA Tyrode's solution were 3.7 ± 1.3 and 7.2 ± 1.7% (N = 4) respectively. No other lysophospholipids were detected in any of these experiments. These latter two values (3.7 and 7.2%) represent levels similar to endogenous lysophospholipids normally present in heart tissue (1.8 ± 0.5 and 4.7 ± 1.7%, N = 5) following incubation *in vitro* for 1 hr. The very low levels of phospholipid hydrolysis (above control endogenous levels) by 5.4×10^{-7} M *N. nigricollis* phospholipase A_2 agree with our previous findings [4–6] and suggest as previously discussed [4–6], that phospholipid hydrolysis is not responsible for the cardiotoxic effects of the *N. nigricollis* enzyme. Higher concentrations could not be used because of the limited amounts of enzyme available.

The ability of *N. nigricollis* phospholipase A_2 to disrupt membrane organization, as shown by its direct hemolytic action [2] and action on plasma lipoproteins [23], may be responsible for the ability of a 100-fold excess of the non-radioactive enzyme to increase binding of the iodinated enzyme. The inability of the *N. n. atra* enzyme to increase binding agrees with its lack of hemolytic action and rela-

Table 1. Phospholipase A_2 (PLA_2) binding to right ventricular heart muscle*

Treatment	Binding of [^{125}I]PLA_2 (pmoles/g heart)	
	N. nigricollis	*N. n. atra*
Normal Tyrode's solution		
None	2.2 ± 0.4 (5)	3.7 ± 1.0 (3)
N. nigricollis PLA_2	4.5 ± 0.9† (5)	—
N. n. atra PLA_2		1.6 ± 0.3 (3)
EDTA (5 mM) Tyrode's solution		
None	3.0 ± 0.6 (4)	
N. nigricollis PLA_2	6.3 ± 1.1‡ (4)	

* The concentration of radioactive phospholipase used was 5.4×10^{-9} M, and the concentration of non-radioactive phospholipases used as treatment agents was 5.4×10^{-7} M. All values are means ± standard errors with the number of experiments shown in parentheses.

† $P < 0.1$.

‡ $P < 0.05$.

tively poor access to phospholipids in organized lipoprotein membranes [2, 23]. The inability of pretreatment with non-radioactive *N. nigricollis* phospholipase A_2 to increase binding of radioactive *N. n. atra* phospholipase A_2 (see unpublished observations previously noted) suggests that the remaining permeability barriers (even after prior exposure to the *N. nigricollis* enzyme) were still formidable for the less penetrating *N. n. atra* enzyme.

To help elucidate whether disruption of permeability barriers exposes additional binding sites to the radioactive enzyme, we checked whether detergents would mimic the action of the *N. nigricollis* enzyme. As shown in Table 2, the cationic detergent cetyltrimethylammonium bromide (CTAB) caused about a 2.5-fold increase in *N. nigricollis* phospholipase A_2 binding, while the other detergents had no significant effect. The results with CTAB are in agreement with previous findings that detergents can increase the permeability of lipid bilayer membranes and can induce the formation of micelles in the lipid bilayer of the cell membrane [24–27]. Alkyltrimethylammonium detergents, such as CTAB, with charged polar groups do not readily diffuse across the lipid bilayer and should preferentially bind to its outer half [24, 25]. This would result in an expansion of the outer half of the membrane and exert an additional disruptive effect upon the bilayer [24, 25] which could then expose more binding sites to *N. nigricollis* phospholipase A_2.

Our previous results (already described) plus the present findings with CTAB suggest that the increased binding of radioactive *N. nigricollis* phospholipase A_2, following exposure to a 100-fold higher concentration of non-radioactive enzyme, was due to disruption of permeability barriers, cellular or extracellular, and exposure to additional binding sites. However, since limitations of material prevented us from carrying out a complete concentration-binding curve, it is possible that the apparent increase in number of binding sites is actually due to a shift of the curve due to increased affinity of binding sites.

In summary, it appears that treatment with a 100-fold excess of non-radioactive *N. nigricollis* phospholipase A_2 exposed additional binding sites to the radioactive *N. nigricollis* enzyme. This disruptive action appears to be independent of phospholipid hydrolysis since it was observed under conditions where little or no hydrolysis occurred and could be mimicked by the cationic detergent cetyltrimethylammonium bromide. These results agree with our previous conclusions, based upon studies with chemically modified derivatives of phospholipase A_2, that the cardiotoxic actions of the *N. nigricollis* phospholipase A_2 are due to a membrane disruptive non-enzymatic action [7, 8].

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Table 2. Detergent effects on binding of [^{125}I]*N. nigricollis* phospholipase A_2 (PLA_2) to ventricular heart muscle*

Treatment	Binding of *N. nigricollis* PLA_2 (% of control)
Cetyltrimethylammonium bromide	270 ± 21† (6)
Lysophosphatidylcholine	144 ± 59 (3)
Sodium desoxycholate	54 ± 32 (2)
Triton X-100	74 ± 24 (3)

* The concentration of radioactive phospholipase used was 5.4×10^{-9} M. All detergents were used in a 1×10^{-3} M concentration in a 5 mM EDTA (0 Ca^{2+}) Tyrode's medium. Mean control value was 4.8 ± 0.6 pmoles/g heart. All values are means ± standard errors with the number of experiments shown in parentheses.
† $P < 0.01$.

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The effect of cicletanide, a diuretic, on the platelet vessel wall interaction; its involvement in the arachidonic acid cascade

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In previous experiments the role of the prostaglandin biochemical pathway in platelet vessel wall interaction was described [1–4] in an *in vivo* model developed in the white male Wistar rat [5, 6]. White platelet thrombus induction and registration was standardized in a branch of the mesenteric artery. Transmission and scanning electronmicroscopic data evidenced that the electrically induced lesion consists of an area of local deendothelialization approximately 150–200 μm in diameter; the surrounding endothelial cells and the smooth muscle cells do not evidence morphological changes while the lamina elastica interna remains intact.

Topical superfusion with ADP (4.10^{-4} M) results in the stimulation of platelet function which results in the adhesion of platelets onto the denuded area followed by aggregation into a thrombotic mass. These phenomena following the stimulation of platelet function depend among other possible mechanisms on the arachidonate cascade occurring in the neighbouring cells, the ratio of the pro-aggregating cyclic endoperoxides to prostacyclin seems the gearing factor [7–9].

More recently, it was evidenced that certain diuretic agents affected the prostaglandin pathway and particularly the generation of prostacyclin [10, 11]. In view of these findings, it was deemed essential to investigate the effect of cicletanide (Fig. 1) in the *in vivo* model in order to evaluate its effect on local ADP induced thrombogenesis. As topical superfusion with cicletanide (10^{-3} M) alone does not affect ADP induced thrombogenesis, the experimental protocol consists then in firstly the registration of control values for the ADP induced thrombi, then the recording of the thrombi when ADP was superfused with tranylcypromine, an inhibitor of prostacyclin synthetase and as such enhances local thrombosis. Secondly control values are again registered then followed by registration of the thrombotic parameters during superfusion with tranylcypromine and cicletanide together.

All experiments are performed with the simultaneous superfusion of ADP with arachidonic acid (10^{-4} M). Tranylcypromine and cicletanide are used at respectively 2.10^{-3} M and 10^{-3} M in order to induce by topical superfusion a sufficient gradient of concentration within the vessel wall. All animals were injected 3 hr previously with 150 mg/kg ASA (lysine salt) intravenously in order to inhibit the platelet cyclooxygenase activity, while the endothelial cell cyclooxygenase activity has recovered completely after this time interval. All solutions were made up in 5% dimethylsulfoxide and the superfusion with tranylcypromine and cicletanide is started 1 min before the ADP superfusion.

Different discriminating parameters are recorded in this investigation: the $m_{(T)}$ value which represents the maximum on the $T(t)$ curve. The $T(t)$ curve represents the sum of all electrical potentials on all LDR elements during each time interval of $30 \times 1/9 \times 10^{-2}$ sec; the $m_{(D)}$ value which represents the maximal on the $D(t)$ curve. The $D(t)$ curve represents at each $30 \times 1/9 \times 10^{-2}$ sec interval the highest potential deviation on one of the LDR elements covered by the thrombus image, the *TTV* value which is the maximal value of the $TTV(t)$ curve resulting from the integration of the $T(t)$ curve in function of time; the *TVM* value which is the value of the $TTV(t)$ curve up to the point where $m_{(T)}$ is reached.

The results are indicated in Table 1.

These findings clearly demonstrate that cicletanide antagonizes the enhancing effect of tranylcypromine on ADP induced thrombogenesis. Previous experimental data [3, 4] demonstrated that the inhibition of PGI_2 synthetase by tranylcypromine markedly enhanced ADP induced thromboformation, a phenomenon which can be explained by the platelet endothelial cell interaction involving cyclic endoperoxides. Cicletanide by itself alone does not modify ADP induced thromboformation; its antagonizing effect on the thrombosis enhancing property of tranylcypromine could be due to some activation of PGI_2 synthetase. Other authors indeed have described this effect in cultures of aortic smooth muscle cells [12] and following the i.v. admin-

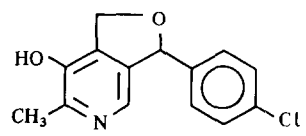

Fig. 1. Cicletanide (BN 1270): 1,3-dihydro 6-methyl 7-hydroxy 3-(4-chlorophenyl) furo (3,4-c) pyridine.